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Zero-valent iron nanoparticles and organic amendment assisted rhizoremediation of mixed contaminated soil using *Brassica napus*

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ABSTRACT

Soil is one of our most important natural resources. Regrettably, the expansion of human activities has resulted in the degradation of the soil resource due to contamination with a myriad of organic and inorganic compounds. The remediation of mixed contaminated soils, i.e. soils contaminated with both organic compounds and metals, is challenging as it requires actions to simultaneously decrease metal-induced risks and organic contaminant concentrations. Here, we evaluated the effect of the addition of zero-valent iron nanoparticles (nanoremediation) and organic amendments (biostimulation) on the rhizoremediation, using *Brassica napus* plants, of soil simultaneously contaminated with zinc (2500 mg kg^{-1}) and lindane (100 mg kg^{-1}). We used a factorial design with three factors (amendment, nZVI, plant) to evaluate the impact of the applied remediation actions on lindane and extractable Zn concentrations, as well as on soil health recovery as manifested by the values of different soil microbial indicators. The studied microbial indicators were not negatively affected by nZVI application. The application of nZVI was the most effective factor regarding the targeted reduction in lindane concentration (51% average reduction in nZVI treated soils). The highest reduction in extractable Zn was achieved in the presence of *B. napus*, nZVI and organic amendments (99 and 95% reduction in horse manure-amended and sewage sludge-amended soils, respectively). The combination of the three factors led to the highest values of soil microbial indicators (although a significant triple interaction was not observed for all parameters), especially when combined with horse manure amendment: in this case, prokaryotic richness increased by 64%, respiration by 376%, eukaryotic abundance by 333%, and prokaryotic abundance by 437%, compared to untreated soils. The combination of remediation approaches (rhizoremediation with *B. napus*, nanoremediation with nZVI, biostimulation with organic amendments) can help overcome the limitations of each individual strategy.

1. Introduction

Soil is one of our most important natural resources as it has a crucial role in the delivery of many ecosystem services (Burges et al., 2016), while harbouring a substantial fraction of the world's biodiversity (Guerra et al., 2020). Soil health

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preservation is currently a crucial challenge facing humanity and, accordingly, a matter of great priority (Garbisu et al., 2017). Regrettably, owing to increased industrial activities over the past decades, many soils are presently contaminated with complex mixtures of both inorganic [e.g., metal(oid)s, trace elements] and organic (e.g., petroleum hydrocarbons, polycyclic aromatic hydrocarbons, solvents, pesticides) compounds (Polti et al., 2014; Volke and Velasco, 2002). It is therefore imperative to urgently deploy cost-effective, environmentally-friendly strategies and solutions to curb soil contamination. Mixed contaminated soils are normally more challenging to remediate (Cameselle and Gouveia, 2019; Polti et al., 2014), as they require specific actions to simultaneously remove/immobilise the toxic metals and degrade the organic contaminants (since, for instance, the presence of potentially toxic metals can hinder the microbial degradation of organic contaminants) (Lacalle et al., 2021). Zinc (Zn) is a potentially toxic metal when present at high concentrations (Dumoulin et al., 2017). In Spain, according to Law 4/2015 for soil contamination prevention and correction (Official State Gazette BOE-A-2015-8272), soil is considered legally contaminated by Zn when its total concentration exceeds tens of grams per kg of dry weight (DW) soil. In turn, lindane is a broad-spectrum organochlorine pesticide (Kumar and Pannu, 2018) included in the catalogue of persistent organic contaminants by the Stockholm Convention (Vijgen et al., 2019). Due to its insecticidal properties, lindane was used during decades for human health and agricultural purposes, leaving a legacy of lindane-contaminated sites. Later, owing to its carcinogenic, bioaccumulative and endocrine disrupting properties, the use of lindane was banned in many countries (Vijgen et al., 2011).

Although there are currently a wide range of soil remediation strategies, many of them (specifically, the physicochemical methods of soil remediation) are often too expensive, environmentally-disruptive (i.e., they habitually damage soil ecosystem integrity), and are only focused on contaminant removal from the soil matrix (Alkorta et al., 2010; Garbisu et al., 2017). Nonetheless, the ideal aspiration of soil remediation strategies is not only to reduce contaminant concentrations (total and/or bioavailable) but also to recover soil health, i.e. the ability of a given soil to perform its function and deliver ecosystem services (Epelde et al., 2009; Burges et al., 2016). The estimation of soil health is still dominated by physicochemical indicators, despite the growing appreciation, during the last decades, of the optimal suitability of biological indicators (Lehmann et al., 2020), particularly microbial indicators (Garbisu et al., 2011; Epelde et al., 2014a,b). Indeed, due to their sensitivity, rapid response and environmental relevance, those microbial parameters that estimate the abundance-biomass, activity and diversity of the soil microbiome are frequently used as bioindicators of soil health (Garbisu et al., 2011; Epelde et al., 2014a,b).

Compared to traditional physicochemical methods of soil remediation, gentle remediation options (GROs) combine a set of remediation technologies that are usually more environmentally-conscious, less invasive and suitable for in situ application in large contaminated sites (Lacalle et al., 2020a; Moreira et al., 2021). Among the GROs, the term rhizoremediation refers to the use of plants and their accompanying rhizosphere microbes to degrade organic contaminants (Barrutia et al., 2011; Hoang et al., 2021). In this phytoremediation strategy, plants stimulate rhizosphere microbial activity through the rhizodeposition of nutrient-containing exudates (Ali et al., 2013; Lacalle et al., 2018). At the same time, rhizosphere microbes can promote plant growth and vigour by enhancing root-growth regulators, increasing mineral uptake and controlling phytopathogens (Praveen et al., 2019). The identification of suitable plant species is key for the success of this phytotechnology. Previous studies have shown the suitability of *Brassica napus* plants for the rhizoremediation of soils contaminated with a mixture of compounds (Aparicio et al., 2021; Lacalle et al., 2018; Míguez et al., 2020), as they can resist high concentrations of soil contaminants (Lacalle et al., 2018). Furthermore, plants from the *Brassicaceae* family can accumulate high concentrations of metals in their aerial parts (Van Ginneken et al., 2007). Relevantly, *B. napus* plants contain lipids in their tissues that can enhance the accumulation potential of chlorinated compounds (Javorská et al., 2009). Finally, *B. napus* is commonly used for biofuel production (Grispen et al., 2006), a fact that adds an economic co-benefit to the use of this species for rhizoremediation purposes, in line with the current phytomanagement concept (Moreira et al., 2021).

Rhizoremediation can be used in combination with other sustainable and economically feasible remediation strategies, such as, for instance, biostimulation (Lacalle et al., 2018). Biostimulation is a remediation strategy focus on the fine-tuning of the environmental conditions (e.g., moisture, aeration, redox potential, pH, temperature) and the application of indispensable nutrients and required electron acceptors, in order to stimulate the activity of degrading microbes and, thereby, decrease soil contaminant concentrations (Garbisu et al., 2017). Moreover, the addition of nutrients helps establish a more propitious environment for plant growth and development, thus increasing rhizoremediation efficiency (Hoang et al., 2021). A potentially more sustainable and cost-effective option for biostimulation is the application of organic wastes as soil amendments (Kanissery and Sims, 2011), a strategy that fits perfectly with the paradigms of waste reuse and circular economy. The addition of organic amendments to soil provides easily-metabolisable carbon and nutrients, as well as microbes, thus stimulating soil microbial populations and promoting plant growth (Hoang et al., 2021), thereby enhancing rhizoremediation efficiency (Adams et al., 2015). In particular, the combination of both strategies (rhizoremediation and biostimulation) has often been employed for the bioremediation of mixed contaminated soils (Aparicio et al., 2021; Lacalle et al., 2018, 2020a).

Zero-valent iron nanoparticles (nZVI) are often used for nanoremediation due to their large surface area to volume ratio (Gómez-Sagasti et al., 2019) and very high reactivity (Anza et al., 2019a). Nanoremediation can target many contaminants, such as organochlorine pesticides, chlorinated organic solvents, polychlorinated biphenyls, etc. (Saccà et al., 2014; Zhao et al., 2016). Earlier studies have reported encouraging results with the combination of GROs and nanoremediation for the recovery of mixed contaminated soils (Aparicio et al., 2021; Lacalle et al., 2018).

The main objective of this study was to investigate, under controlled microcosm conditions, the effect of the application of nZVI (nanoremediation) and organic amendments (biostimulation with composted horse manure *versus* composted sewage sludge) on the rhizoremediation with *Brassica napus* plants of a soil simultaneously contaminated with lindane and Zn. A factorial design (three factors: plant, nZVI, amendment) was used to study the hypothesised beneficial effects of the combination of the abovementioned remediation strategies (rhizoremediation, nanoremediation and biostimulation) on: (i) the degradation of soil lindane; (ii) the reduction of soil extractable Zn concentrations; and (iii) the recovery of soil health.

2. Materials and methods

2.1. Experimental design

One hundred kilograms of clay loam topsoil (0–30 cm, pH = 5.49; organic matter-OM content = 2.90%; C/N = 9.2) was collected from a non-contaminated grassland. The collected soil was dried at 30 °C, manually homogenised and mechanically sieved (<6 mm). The soil was then artificially contaminated with lindane and Zn. Lindane (γ-hexachlorocyclohexane) (Sigma-Aldrich, St. Louis, Missouri, USA) was applied to the soil in an n-hexane/acetone (50/50 v/v) solution to achieve a concentration of 100 mg lindane kg^{-1}DW soil. Zinc contamination was accomplished by adding zinc nitrate hexahydrate – $Zn(NO_3)_2.6H_2O$ – powder (Sigma-Aldrich) to a water solution in order to obtain a concentration of 2500 mg Zn kg^{-1} DW soil. All the soil was contaminated at the same time using a cement mixer and, subsequently, stored for one month to allow the contaminants to interact with the soil matrix. After this one-month ageing period, commercial nZVI slurry (see below) was mixed with 50 kg of the previously contaminated soil at a 1% (w/w) concentration. After one week, and in order to improve nanoremediation efficiency, a second addition of 1% nZVI was performed to achieve a final concentration of 2% (w/w), following Anza et al. (2019a). Commercial NANOFER 25S was obtained from NANO IRON s.r.o. company, Czech Republic, as slurry of stabilised-coated nZVI (20% nZVI, 77% water, 3% organic stabiliser; average nZVI size <50 nm).

Afterwards, both nZVI-treated and nZVI-untreated soils were divided into three blocks and two of them were individually amended with composted horse manure or composted sewage sludge, making a total of three amendment treatments: unamended soil (non-amended control), horse manure-amended soil (horse) and sewage sludge-amended soil (sewage). Composted horse manure (pH = 9.1; OM content = 37%; C/N = 22) was provided by Bolaleku S.A.T (Spain). Composted sewage sludge (pH = 8.1; OM content = 45%; C/N = 11) was kindly provided by Arazuri Wastewater Treatment Plant (Pamplona, Spain). The physicochemical characterisation of both amendments is shown in Table 1. Amendment doses were adjusted to achieve a soil OM content of 10%, starting from the initial 5.8% present in the unamended soil.

Experimental pots containing 4 kg of soil were placed in a growth chamber under the following conditions: 25/22 °C day/night, relative humidity = 65%, and 300 μmol photons m^{-2} s^{-1}, using a 14 h photoperiod. *Brassica napus* L. seeds (20 seeds per pot) were sown in half of the pots. After seed germination, only six homogeneous plants were left in each pot (the others were removed). Then, the following twelve treatments were studied: *Brassica*/No nZVI/Unamended; *Brassica*/No nZVI/Horse; *Brassica*/No nZVI/Sewage; *Brassica*/nZVI/Unamended; *Brassica*/nZVI/Horse; *Brassica*/nZVI/Sewage; Unplanted/No nZVI/Unamended; Unplanted/No nZVI/Horse; Unplanted/No nZVI/Sewage; Unplanted/nZVI/Unamended; Unplanted/nZVI/Horse; and Unplanted/nZVI/Sewage. Four independent replicates were used for each treatment (i.e., a total of 48 pots).

Plants were grown for 16 weeks and bottom watered periodically as needed. Plants and soils were sampled at the end of the abovementioned 16-week period. Plant shoots were cut, oven-dried at 70 °C during 48 h, and their dry weights and biomass measured. Soils were sampled and sieved (<2 mm).

2.2. Determination of soil physicochemical properties

Soil texture was analysed by laser diffractometry using a Mastersizer 2000 system. Soil organic matter content was determined following Nelson and Sommers (1996). Total nitrogen (N) was determined by combustion with a TruSpec CHN analyser (LECO Corporation, Michigan, USA) according to ISO 13878 Norm (1998). Soil pH was quantified in water at 1:2.5 w/v. Olsen phosphorus (P) was measured following Watanabe and Olsen (1965). Cation exchange capacity and extractable potassium (K^{+}) content were measured following MAPA (1994).

With respect to soil contaminants, extractable Zn concentrations were measured, according to Houba et al. (2000), with 0.01 M calcium chloride ($CaCl_2$) as extraction reagent. Zinc concentrations were determined by digestion in a mixed acid solution (HNO_3 1% + $HClO_4$ 2%), following Zhao et al. (1994), and then quantified using an ICP-AES spectrophotometer. Soil lindane concentrations were measured by gas chromatography [n-hexane/acetone (50/50 v/v) extraction], following Anza et al. (2018).

Table 1
Physicochemical characterisation of the organic amendments.

Parameter	Unit (dry weight)	Composted horse manure	Composted sewage sludge
pH	–	9.1	8.1
Conductivity	$\mu S\ cm^{-1}$	–	3690
Organic matter	%	37	45
C/N	–	22	11
Total N	%	0.2	2.0
P	$g\ kg^{-1}$	1.8	9.0
K	$g\ kg^{-1}$	7.1	8.7
Mg	$g\ kg^{-1}$	4.7	4.1
Na	$g\ kg^{-1}$	1.3	<3.0
Fe	$g\ kg^{-1}$	12.0	7.4
Mn	$mg\ kg^{-1}$	161	191
Cu	$mg\ kg^{-1}$	27	66
Zn	$mg\ kg^{-1}$	62	273

2.3. Determination of soil microbial properties

For the analysis of microbial parameters, sampled soils were stored at 4 °C for a maximum of two months until analysis. For molecular analyses, soil samples were stored at −20 °C. β-D-glucosidase (GLU, EC 3.2.1.21), glucosaminidase (GLM, EC 3.2.1.30), xylosidase (XIL, EC 3.2.1.37), phosphatase (PHO, EC 3.1.3.2), sulphatase (SUL, EC 3.1.6.1) and L-Leu-aminopeptidase (LAP, EC 3.4.11.1) enzyme activities were measured according to ISO/TS 22939 Norm (2010). These enzymes activities were selected as they play important roles in the main (C, N, P, S) biogeochemical cycles (Kelley et al., 2011). Potentially mineralisable nitrogen (N_{PM}) and soil respiration were measured to estimate soil microbial activity. Potentially mineralisable nitrogen was quantified following Powers (1980). Soil basal respiration was measured according to ISO 16072 Norm (2002). Microbial biomass carbon (C_{MB}) was determined following the fumigation–extraction method of Vance et al. (1987).

For molecular analyses, 250 mg of soil was washed twice with 120 mM K_2PO_4 (pH = 7.8) (Kowalchuk et al., 1997). Soil DNA was extracted with the PowerSoil™ Isolation Kit (MO Bio Laboratories, CA, USA). DNA concentrations were quantified using TapeStation 4200 (Agilent Technologies). The total abundance of 16S rRNA (prokaryotes, 424bp) and 18S rRNA (eukaryotes, 636bp) gene fragments was measured by real-time PCR (qPCR) (Epelde et al., 2014a). The primers used for the amplification of 16S rRNA gene were Ba519f (CAGCMGCCGCGGTAANWC) and Ba907r (CCGTCAATTCMTTTRAGTT), with an annealing temperature of 50 °C. The primers used for the amplification of the 18S rRNA gene were Fung5f (GGG GAA CCA GGA CTT TTA C) and FF390r (AGG TCT CGT TCG TTA TCG), with an annealing temperature of 52 °C. Each analysis was carried out in triplicate.

To study the impact of remediation actions on the structure of prokaryotic communities, amplicon libraries were prepared, according to a dual indexing approach, with sequence-specific primers targeting the V4 region of the 16S rRNA gene (Lanzén et al., 2016; Urra et al., 2019). In the first amplification step, adapter-linked 519F (CAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) primers were used in a volume of 20 μL (Lanzén et al., 2016). Amplicon libraries were cleaned with AMPure XP (Beckman Coulter Genomics). In the second amplification step (10 cycles), barcoded primers were used in a volume of 50 μL (Lanzén et al., 2016). Sequencing was performed using Illumina MiSeq with V2 Kit at Tecnalia, Spain. Read pairs were quality filtered, overlapped, and clustered into OTUs (operational taxonomic units) according to Lanzén et al. (2016). In order to taxonomically classify OTUs, the obtained sequences were aligned (SilvaMod database) using BLASTn, and thereafter classified with CREST following Lanzén et al. (2012). Amplicon sequencing data were submitted to the European Nucleotide Archive (accession number: PRJEB44356).

2.4. Statistical analysis

The influence of the three experimental factors (*B. napus* presence, nZVI, organic amendment) and their interactions on physicochemical and microbial parameters were evaluated using a three-way ANOVA with SPSS Statistics 18.0.0 software (SPSS, Chicago, IL, USA). When double or triple interactions between factors were significant ($p < 0.05$), significant differences were tested using Duncan's test. Redundancy analyses (RDA) were used to explore treatment effects on soil parameters using CANOCO 5.0 (ter Braak and Smilauer, 2012). The R package vegan (Oksanen et al., 2015) and Rcmdr library (Field et al., 2012) were used for the visualisation of 16S rRNA amplicon sequencing data and the calculation of biodiversity indices. Rarefied richness estimates (obtained by means of interpolating the expected richness at the lowest sample-specific sequencing depth) were used in order to compensate for variations in read numbers across soil samples. The Decostand function was used to transform the distributions of OTUs into relative abundance values. A permutational analysis of variance (PERMANOVA) was carried out to determine the effect of treatments on microbial community composition using the adonis function. Based on Bray Curtis dissimilarities, a hierarchical clustering dendrogram of OTUs was performed.

3. Results

3.1. Soil contaminants

Lindane contamination was reduced in all treatments (initial concentration was 100 mg lindane kg^{-1} DW soil; all treatments showed concentrations $<$ 33 mg kg^{-1}DW soil at the end of the experiment) (Fig. 1A). Regarding lindane concentrations in soil, the three-way ANOVA showed a significant triple interaction among the three studied factors (Supplementary Table 1). A maximal decrease in soil lindane concentration was observed for Brassica/nZVI/Unamended treatment, with a concentration value 66% lower than that observed in the least efficient treatment (i.e., Brassica/No nZVI/Sewage) (Fig. 1A). Nevertheless, no significant differences were observed between Brassica/nZVI/Unamended, Brassica/nZVI/Horse and Brassica/nZVI/Sewage treatments. Actually, lindane concentration was significantly lower in the two latter treatments (59% lower in both cases), compared to the treatment which showed the highest lindane concentration (i.e., Brassica/No nZVI/Sewage) (Supplementary Table 1). In any case, the experimental factor that showed the greatest effect on lindane concentration was nZVI application (actually, lindane concentration was reduced by an average of 51% in nZVI-treated soils, compared to nZVI-untreated soils).

On the other hand, the interaction among the three studied factors significantly reduced soil extractable Zn concentration (Supplementary Table 1). The untreated control soil (Unplanted/No nZVI/Unamended) presented the highest extractable Zn concentration (Fig. 1B), while the most significant reduction was detected in organically amended soils in the presence of B. napus plants and nZVI. Higher values of extractable Zn were found in sewage sludge-amended versus horse manure-amended soils (Fig. 1B), which was most likely related to the higher levels of Zn initially present in the latter amendment (Table 1). Total Zn concentrations were significantly lower only in planted soils: total Zn was reduced by an average of 11% in planted versus unplanted soils (Supplementary Table 1).

3.2. Soil physicochemical properties

The effect of the studied treatments on soil physicochemical parameters is shown in Fig. 2. Data on the effects of the experimental factors and their interactions on soil physicochemical parameters are shown in Supplementary Table 2. The absence of B. napus was positively linked with the increase in total N, extractable K^{+}, Olsen P and OM content towards the negative region of RDA-1 (this axis explained 66% of the variance) (Fig. 2). The application of nZVI significantly increased pH and CEC. The application of amendments (both composted horse manure and composted sewage sludge) had the greatest effect on soil physicochemical parameters, as observed along RDA-1 (Fig. 2). In general, both amendments increased the values of the studied soil physicochemical parameters, compared to unamended soils. The observed increase in CEC, total N and extractable K^{+} was higher in horse manure-amended soil, whereas Olsen P was higher in sewage sludge-amended soil. The increase in OM content was similar in both amended soils. A statistically significant increase in pH values was also observed in amended versus unamended soils, especially when composted horse manure was added (these higher pH values are probably related with the lower extractable Zn concentrations detected in the amended soils).

3.3. Soil microbial properties

Fig. 3A shows the influence of the studied experimental factors on microbial activity parameters. Xylosidase and glucosaminidase activities were positively linked with the presence of B. napus plants, while phosphatase and β-D-glucosidase activities were negatively linked with such presence. Values of N$_{PM}$, respiration, and sulphatase and L-Leu-aminopeptidase activities increased in the presence of both nZVI and organic amendments (both amendments significantly increased respiration and N$_{PM}$) (Supplementary Table 3). The highest respiration values were detected in B. napus/nZVI/Horse treatment, while the largest increase in N$_{PM}$ occurred in B. napus/nZVI/Sewage treatment (here it must be taken into consideration that the composted sewage sludge amendment had 10 times more nitrogen than the composted horse manure amendment) (Table 1).

Fig. 3B shows the influence of the studied experimental factors on microbial biomass parameters. Microbial biomass values increased towards the left side of RDA-1 (this axis explained 42% of the variance), where the experimental factors (plant, nZVI, amendment) were located. However, values of C$_{MB}$ decreased by 33% in the presence of B. napus, compared to unplanted treatments (Supplementary Table 4). In contrast, the application of nZVI led to 24% higher C$_{MB}$ values compared to no-nZVI treatments, while the application of horse manure and sewage sludge increased the values of this parameter by 51 and 38%, respectively, compared to unamended treatments. Prokaryotic abundance (quantified by qPCR) increased by 18% in planted versus unplanted soils. Similarly, a significant interaction ($p < 0.001$) was observed between nZVI and amendment factors, with highest prokaryotic abundance values being observed when nZVI application was combined with the addition of horse manure (372%) and sewage sludge (216%), compared to No nZVI/Unamended treatments, irrespective of the presence of B. napus. Although no significant triple interaction between factors was observed, the highest prokaryotic abundance was found in Brassica/nZVI/Horse treatment, with an increase of 437% compared to untreated soil (i.e. Unplanted/No nZVI/Unamended) (Supplementary Table 4). Regarding eukaryotic abundance, a significant interaction ($p < 0.01$) was observed between plant and nZVI factors. Eukaryotic abundance increased by 196% in the presence of B. napus plants and nZVI, in comparison to Unplanted/No nZVI treatments, irrespective of the presence of amendments. In
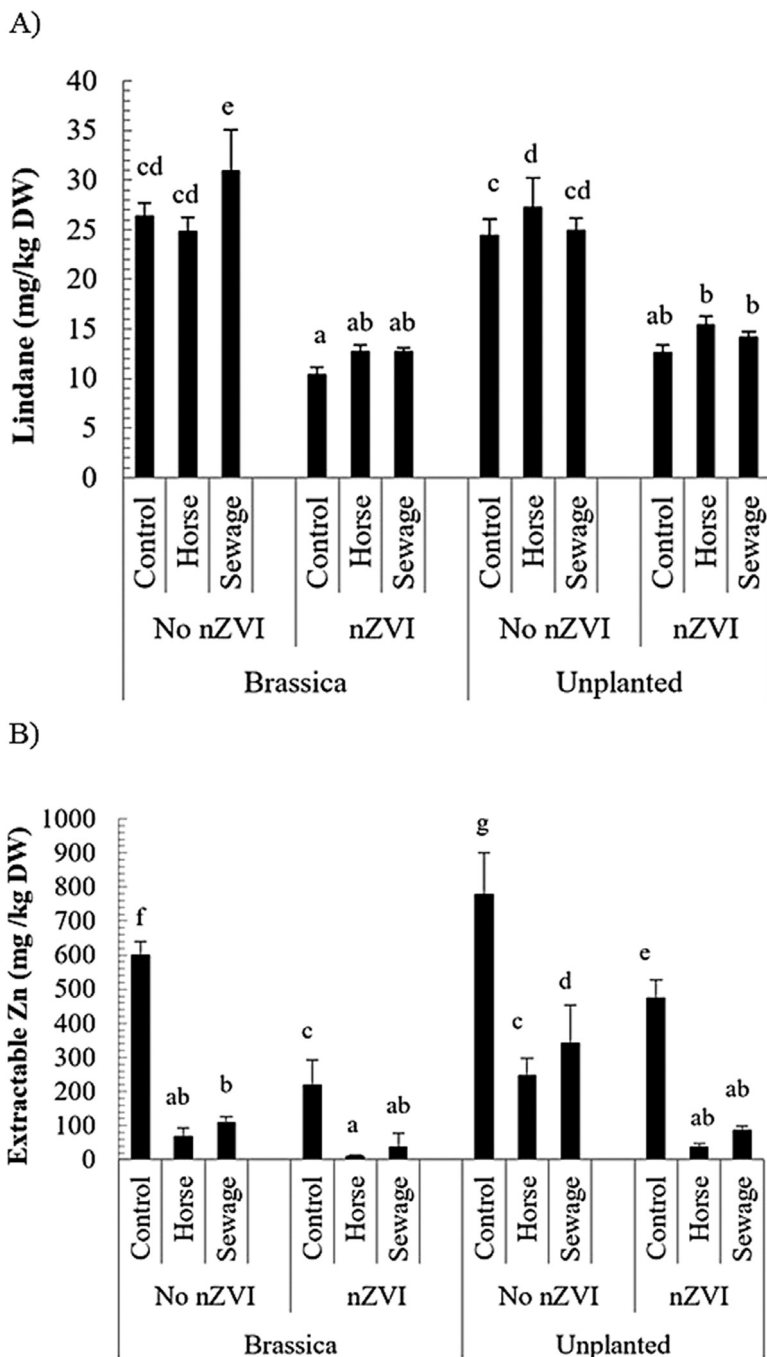
A)

B)

Fig. 1. Effect of treatments on soil (a) lindane and (b) extractable Zn concentrations. Mean values ($n = 4$) $\pm$ standard deviation. Treatments with different letters are significantly different ($p < 0.05$) according to Duncan's test. Control: unamended treatment.

addition, the application of horse manure and sewage sludge increased eukaryotic biomass by 81 and 69%, respectively, compared to unamended soil. As with prokaryotic abundance, no significant triple interaction between factors was observed regarding eukaryotic abundance, but the highest value was also detected in *Brassica*/nZVI/Horse treatment, with an increase of 333%, compared to untreated soil (Supplementary Table 4).

Pertaining to the composition of microbial (bacterial) communities, plant and nZVI factors did not show any statistically significant effect (Fig. 3C). In fact, the distribution of the 30 most abundant bacterial families was predominantly based on the amendment factor, which showed a significant effect ($p < 0.001$) on all families, except for *Sphingobacteriaceae* which
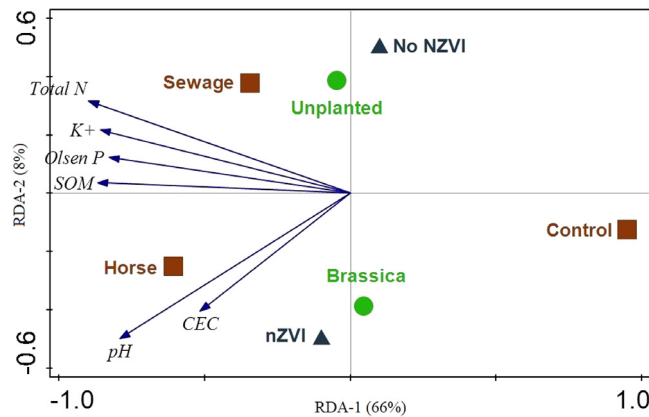
Fig. 2. Biplot of the redundancy analysis carried out on the experimental factors as explanatory variables (symbols) and physicochemical parameters as response variables (arrows). N: total nitrogen; K: potassium; P: Olsen phosphorous; OM: organic matter content; CEC: cation exchange capacity.

Table 2
Influence of treatments on soil prokaryotic diversity indexes.

			Shannon	Simpson	Pielou	Richness
Brassica	No nZVI	Control	4.9 ± 0.2	0.97 ± 0.01	0.64 ± 0.02	2099 ± 190
		Horse	5.6 ± 0.2	0.99 ± 0.00	0.71 ± 0.02	2669 ± 316
		Sewage	5.6 ± 0.1	0.99 ± 0.00	0.72 ± 0.01	2469 ± 66
	nZVI	Control	5.0 ± 0.1	0.98 ± 0.01	0.65 ± 0.02	2167 ± 100
		Horse	5.7 ± 0.1	0.99 ± 0.00	0.71 ± 0.02	2794 ± 56
		Sewage	5.8 ± 0.1	0.99 ± 0.00	0.73 ± 0.01	2771 ± 222
Unplanted	No nZVI	Control	4.5 ± 0.1	0.97 ± 0.00	0.60 ± 0.01	1708 ± 43
		Horse	5.4 ± 0.1	0.98 ± 0.00	0.68 ± 0.01	2754 ± 98
		Sewage	5.3 ± 0.1	0.98 ± 0.00	0.67 ± 0.01	2513 ± 148
	nZVI	Control	4.8 ± 0.2	0.97 ± 0.01	0.63 ± 0.02	2120 ± 151
		Horse	5.6 ± 0.1	0.99 ± 0.00	0.71 ± 0.01	2714 ± 162
		Sewage	5.6 ± 0.2	0.98 ± 0.00	0.70 ± 0.02	2689 ± 222
Plant			***	***	***	ns
nZVI			***	***	***	***
Amendment			***	*	***	***
Plant * nZVI			ns	ns	ns	ns
Plant * Amendment			ns	ns	*	ns
nZVI * Amendment			ns	ns	ns	ns
Plant * nZVI * Amendment			ns	ns	ns	ns

Mean values ($n = 4$) $\pm$ SD. Probability values from three-way ANOVA for the effects of "plant", "nZVI", "amendment" and their interactions are shown. Control: unamended soil; Horse: soil amended with composted horse manure; Sewage: soil amended with composted sewage sludge; ns: non-significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

was more affected by nZVI application (Supplementary Table 5). Some bacterial families showed different abundance values depending on the type of amendment used (Fig. 3C). *Alcaligenaceae, Comamonadaceae* and *Bradyrhizobiaceae* were more abundant in unamended soils. Instead, *Cytophagaceae*, *Flavobacteriaceae* and *Hyphomicrobiaceae* were found more abundant in composted horse manure-amended soils, whereas *Mycobacteriaceae*, *Phyllobacteriaceae* and *Bacillaceae* were more abundant in composted sewage sludge-amended soils. The hierarchical clustering (Fig. 4) showed a well-defined division in prokaryotic OTUs according to the amendment factor: unamended *versus* both amendments. The next level of clustering was found between the nZVI factor in unamended soils and between horse manure-amended and sewage sludge-amended soils. The third level of clustering in amended soils corresponded to the nZVI factor. Finally, the plant factor had the smallest effect on soil bacterial composition.

Regarding prokaryotic diversity indices (Table 2), no significant interactions between factors were found, except for Pielou index which showed a significant ($p < 0.05$) interaction between plant and amendment factors: Pielou index increased by 17% in the presence of *B. napus* and sewage sludge amendment, and by 16% in the presence of *B. napus* and horse manure amendment, compared to Unplanted/Unamended soil, irrespective of the presence of nZVI. In general, each factor had a significant effect on Shannon and Simpson indices, showing higher values for *B. napus*, nZVI and amendment factors, compared to their respective controls (unplanted, no-ZVI, unamended). The greatest effect on Shannon index was observed in amended treatments, showing an increase of 16% in both horse manure-amended and sewage sludge-amended treatments, compared to unamended controls. Likewise, regarding prokaryotic richness, although a significant
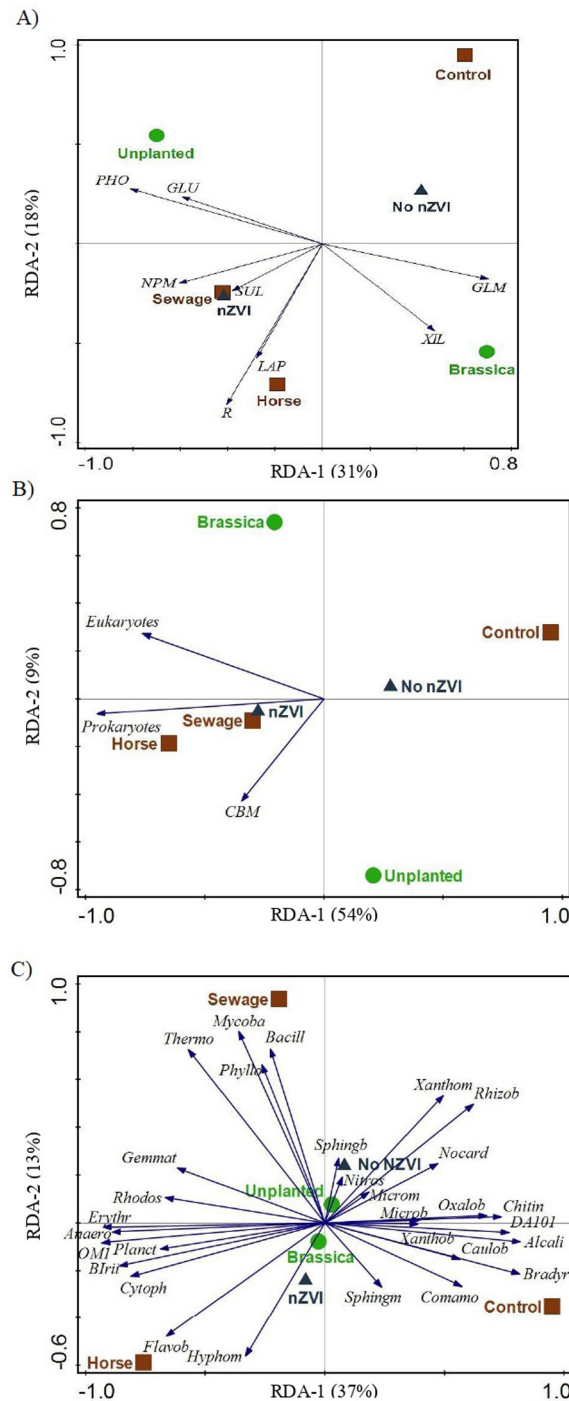
Fig. 3. Biplots of the redundancy analysis performed on the experimental factors as explanatory variables (symbols) and soil microbial (a) activity, (b) biomass, and (c) taxonomic composition at family level (30 most abundant families) as response variables (arrows). R: soil respiration; NPM: potentially mineralisable nitrogen; GLU: β-D glucosidase; GLM: glucosaminidase; XYL: xylosidase; SUL: sulphatase; PHO: phosphatase; LAP: L-Leu-aminopeptidase; CMB: microbial biomass carbon; Eukaryotes: eukaryotic abundance; Prokaryotes: prokaryotic abundance; *Xanthom: Xanthomonadaceae; Chitin: Chitinophagaceae; Cytoph: Cytophagaceae; Sphingm: Sphingomonadaceae; Alcali: Alcaligenaceae; Bradyr: Bradyrhizobiaceae; Hyphom: Hyphomicrobiaceae; Flavob: Flavobacteriaceae; Sphingb: Sphingobacteriaceae; Caulob: Caulobacteraceae; Planct: Planctomycetaceae; Oxalob: Oxalobacteraceae; Xanthob: Xanthobacteraceae; Gemmat: Gemmatimonadaceae; Phyllo: Phyllobacteriaceae; Rhizob: Rhizobiales Incertae Sedis; Anaero: Anaerolineaceae; Microb: Microbacteriaceae; Rhodos: Rhodospirillaceae; Thermo: Thermomonosporaceae; Bacill: Bacillaceae; Norcad: Nocardioidaceae; Nitros: Nitrosomonadaceae; OM1: OM1 clade; Comamo: Comamonadaceae; Mycoba: Mycobacteriaceae; DA101: DA101 soil group; Micром: Micromonosporaceae; Erythr: Erythrobacteraceae; Blrii: Blrii41.*
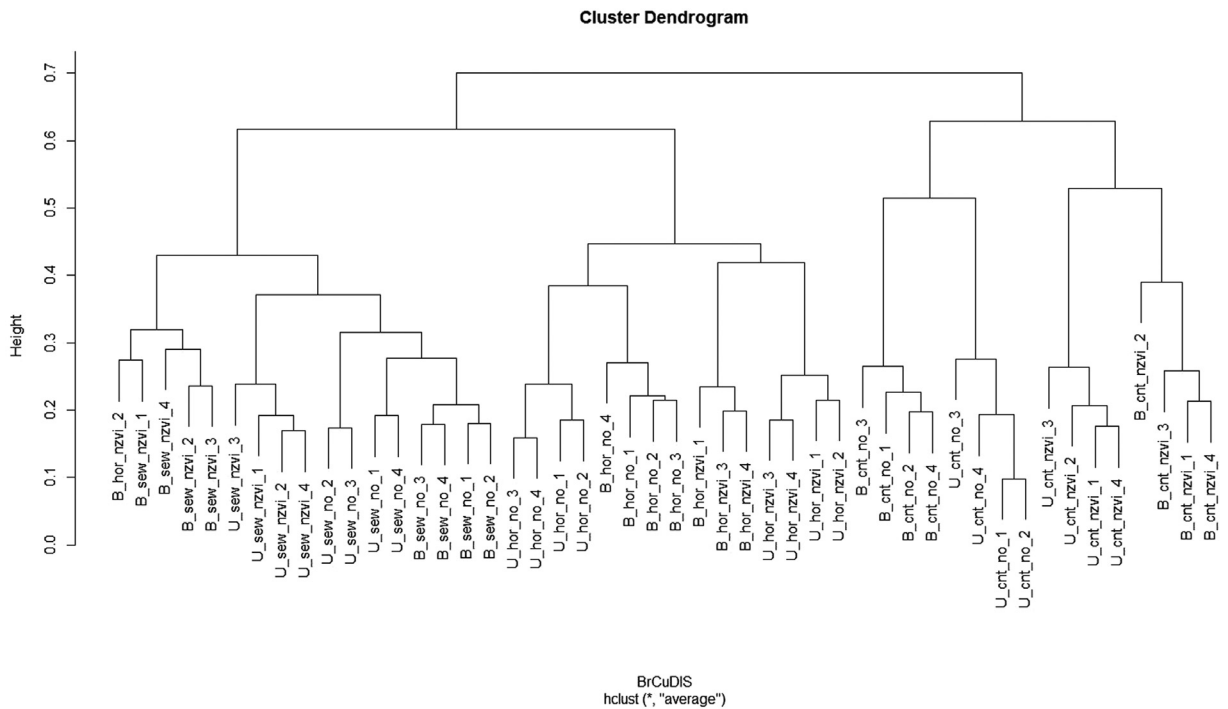
Cluster Dendrogram

Fig. 4. Hierarchical clustering of soil samples, based on Bray Curtis dissimilarities of prokaryotic OTUs (16S rRNA metabarcoding). B: *Brassica napus*; U: unplanted; no: nZVI-untreated soil; zvi: nZVI-treated soil; cnt: unamended control; hor: soil amended with composted horse manure; sew: soil amended with composted sewage sludge.

Table 3
Influence of treatments on *B. napus* biomass.

Treatments		Plants (No.)	Plant weight per pot (g DW)	Individual plant weight (g DW)
no nZVI	Control	0^c	0.0 ± 0.0	0.0 ± 0.0
	Horse	4^c	4.1 ± 3.9	3.0 ± 2.2
	Sewage	6^{bc}	5.4 ± 3.5	3.3 ± 1.1
nZVI	Control	13^a	3.3 ± 2.1	1.0 ± 0.3
	Horse	12^{ab}	21.0 ± 8.9	7.8 ± 5.1
	Sewage	6^{bc}	14.0 ± 11.0	9.2 ± 6.9
nZVI		***	**	*
Amendment		ns	**	**
nZVI * Amendment		*	ns	ns

Mean values ($n = 4$) ± SD Probability values from two-way ANOVA for the effects of "nZVI", "amendment" and their interaction are shown. When double interaction is significant ($p < 0.05$), means with different letters represent significant differences among treatments, according to Duncan's test. Control: unamended soil; Horse: soil amended with composted horse manure; Sewage: soil amended with composted sewage sludge; ns: non-significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

triple interaction between factors was not observed, the highest value was found in *Brassica*/nZVI/Horse treatment, with an increase of 64%, compared to untreated soil (Table 2).

3.4. Plant parameters

No plant growth was observed in untreated soils that were not subjected to nZVI or amendment application (Table 3). The application of nZVI significantly increased plant survival and biomass, compared to no-nZVI treatments. Similarly, the application of amendments significantly enhanced plant survival and biomass. The combination of nZVI and amendments increased plant biomass by 7.8 and 9.2-fold for horse manure and sewage sludge amendment, respectively, compared to unamended soils, although no significant differences were found between the two amendment treatments themselves. Interestingly, as indicated above, nZVI-treated soils presented the lowest levels of extractable Zn, which could explain the higher plant biomass values found in nZVI-treated soils.

4. Discussion

In the absence of any treatment, soil lindane concentration was reduced by more than 70% from the beginning to the end of the experiment. Different processes such as contaminant volatilisation (Haenel and Siebers, 1995) and biodegradation by native microorganisms (Aparicio et al., 2021) can have contributed to such decrease in lindane concentration (Fig. 1A). Among the studied factors (i.e., plant, nZVI, amendment), the application of nZVI was the most effective factor in reducing soil lindane concentration. The predominant mechanism for this nZVI-induced lindane removal is reductive dehalogenation (Singh et al., 2011), which occurs through the directional transfer of electrons from nZVI to the contaminant, thus transforming it into a non-toxic or less toxic compound (Dominguez et al., 2018).

The simultaneous presence of plant, nZVI and amendment led to the highest reduction in soil extractable Zn, being the application of amendments the factor that had the most relevant contribution. This is in line with data reported by Grobelak et al. (2017) and Pérez-Esteban et al. (2012), who found that the addition of horse manure and sewage sludge compost significantly reduced soil metal bioavailability, while increasing soil CEC. The organic amendment-induced retention of metals in the soil matrix can be, at least partly, attributed to an increase in the surface charge and the occurrence of metal binding compounds (Park et al., 2011). Furthermore, the increase in soil pH observed in organically amended soils (Supplementary Table 2) can also have contributed to the observed decrease in extractable Zn and, concomitantly, to the improvement of soil health as manifested by the values of soil microbial indicators (Galende et al., 2014). The large surface area to volume ratio of nZVI, a property that strongly drives the adsorption of compounds onto nanoparticles surfaces (Gómez-Sagasti et al., 2019), can also have promoted the reduction of extractable Zn in nZVI-treated soils. In the same way, the presence of *B. napus* plants might have decreased soil extractable Zn concentrations via root sorption, precipitation and complexation in the rhizosphere (Ali et al., 2013; Park et al., 2011).

In general, an increase in soil microbial activity (enzyme activities, respiration, N_{PM}), biomass (C_{MB}, prokaryotic and eukaryotic abundance), and diversity were observed in the simultaneous presence of the three (plant, nZVI, amendment) factors. Soil micro(biological) properties are relevant, sensitive, practical and informative indicators of soil health (Lehmann et al., 2020) and, therefore, they have recurrently been used as bioindicators of the adverse effects of contaminants, as well as of the beneficial effects of soil remediation treatments, on soil health (Barrutia et al., 2011; Burges et al., 2016; Epelde et al., 2014b; Lacalle et al., 2021). The observed recovery of soil health may be partly related to the reduction of contaminant concentrations that was detected in the presence of the studied factors (Fig. 1), but also to the improvement of soil physicochemical conditions derived from the application of treatments (especially, organic amendments and plant growth). According to Dotaniya et al. (2019), soil enzyme activities are influenced by physicochemical properties, such as pH, OM content, nutrient levels, texture and electrical conductivity. Soil physicochemical properties control nutrient availability for microbes, a fact which is then reflected in the values of microbial activity parameters (Fang et al., 2010; Sinsabaugh et al., 2005). Here, *B. napus* growth resulted in a decrease of soil total N, extractable K^+ and Olsen P, which could be related to plant uptake, as these elements are essential nutrients for plant growth and development. Finally, the application of organic amendments led to an increase in pH (bringing it closer to neutral values), OM content and nutrients, compared to unamended controls.

As mentioned above, no plant growth was observed in untreated soils that were not subjected to nZVI or amendment application. This lack of *B. napus* growth was possibly due to the phytotoxicity imposed by the high levels of extractable Zn present in untreated soils (Supplementary Table 1). This underlines the need for the application of nZVI and/or organic amendments to promote plant growth and development in mixed contaminated soils similar to ours when subjected to rhizoremediation (Anza et al., 2019b). Furthermore, the presence of *B. napus* increased soil eukaryotic biomass and prokaryotic biodiversity, possibly due to root growth and exudation (Sparks et al., 2011).

In agreement with Lacalle et al. (2020b), microbial parameters were not negatively affected by nZVI application. Nonetheless, other studies have reported adverse effects of nZVI on the microbial communities of mixed contaminated soils, with no changes being detected in the same non-contaminated soil, possibly due to an "stress on stress" effect (Anza et al., 2019a). Lacalle et al. (2018) found that the application of nZVI had no effect on soil microbial parameters, but it did cause an indirect negative impact on plant root elongation, possibly due to the interaction of nZVI with soil OM. In any case, many studies have concluded that the negative or positive effects of nZVI on soil health can vary significantly depending on the soil's physicochemical properties (Fajardo et al., 2018; Saccà et al., 2014; Gómez-Sagasti et al., 2019; Fujioka et al., 2016; Vítková et al., 2017). Fajardo et al. (2018) observed changes in soil autochthonous bacterial communities at the phylogenetic level, but not at the functional level, and concluded that the overall functionality of the ecosystem was not affected by nZVI. Nevertheless, their elevated price makes the use of nZVI an unfeasible technique for the nanoremediation of large contaminated areas. Furthermore, despite promising results in the nanoremediation of groundwater and wastewater (Li et al., 2014; Dong et al., 2015; Shi et al., 2015; Sun et al., 2017; Tosco et al., 2014; Zhang and Elliott, 2006), nanoparticle homogenisation and distribution in the soil matrix is extremely complicated because of their high reactivity, which can prevent them from reaching the target contaminants. In this respect, strong attractive forces among nZVI induce nanoparticle agglomeration, thus forming microsized aggregates and, hence, reducing their mobility and applicability in the soil matrix (Qian et al., 2020). This low colloidal stability strongly hinders their application in the field.

As stated above, the addition of organic amendments resulted in the greatest effects on soil microbial activity (Supplementary Table 3), biomass (Supplementary Table 4), and prokaryotic diversity (Supplementary Fig. 1). Garau et al.
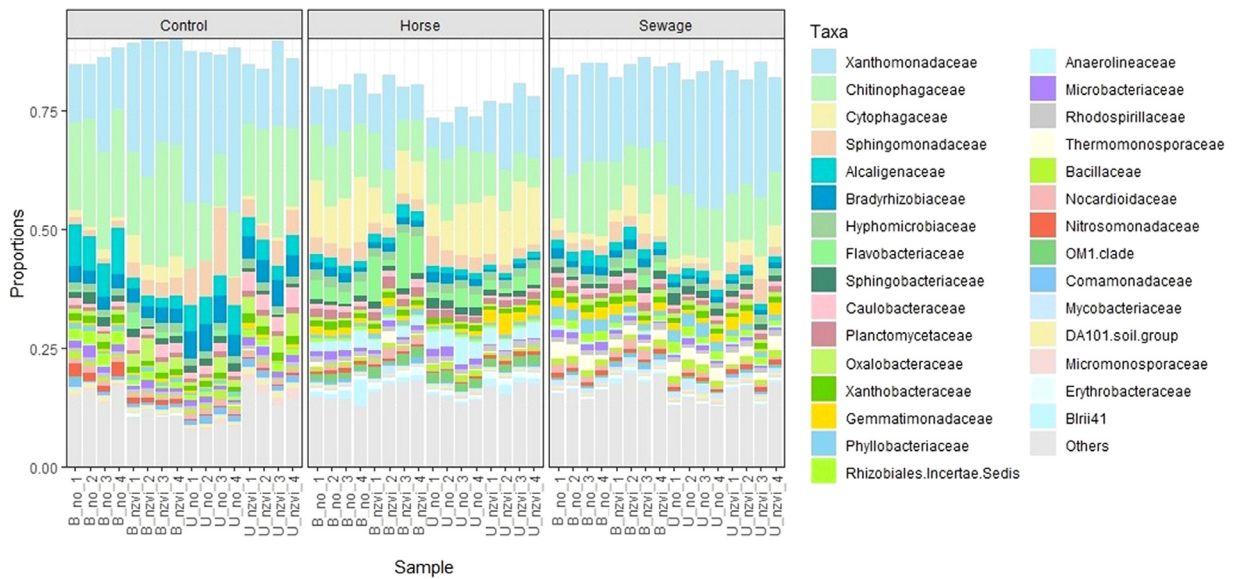
Fig. 5. Barplots of the relative abundance values of the 30 most abundant bacterial taxa (family level), grouped by amendment treatments. B: *Brassica napus*; U: unplanted; no: nZVI-untreated soil; zvi: nZVI-treated soil.

(1986) reported an increase in N_{PM} as a result of the application of sludge. Similarly, Liu et al. (2007) observed that the application of organic amendments increased microbial diversity in the studied soil. Many remediation studies have reported the stimulatory effect of organic amendments on soil microbial abundance-biomass, activity and biodiversity (Lacalle et al., 2020a, 2021; Galende et al., 2014; Garaiyurrebaso et al., 2017).

Concerning the composition of soil microbial communities, the main significant differences were also due to amendment treatment (Fig. 5). A greater abundance of *Alcaligenaceae, Comamonadaceae* and *Bradyrhizobiaceae* was detected in the unamended soils. Members of these families have previously been described as degraders of organic contaminants, such as BTEX, TPHs, glyphosate, PAHs, fuel oil and diesel, and as key players in the bioremediation of organically contaminated sites (Ermakova et al., 2010; Jones et al., 2011; Wang et al., 2016). Lima-Morales et al. (2016) found that members of *Alcaligenaceae* and *Comamonadaceae* were enriched in soil during incubation with benzene. Galazka et al. (2018) found that members of *Bradyrhizobiaceae* and *Sphingomonadaceae* were dominant in soils collected from oil wells. These findings suggest that the abovementioned three bacterial families have a great tolerance to a wide range of organic contaminants, which could, at least partly, explain their higher abundance in unamended soils, where contaminant (lindane) concentrations were significantly higher. In those soils amended with composted horse manure, the dominant families were *Cytophagaceae, Flavobacteriaceae* and *Hyphomicrobiaceae*. Anza et al. (2019a) also found *Cytophagaceae* as one of the dominant families in those soils amended with composted horse manure. Similarly, the abundance of *Cytophagaceae* family also increased in horticultural soil fertilised with organic amendments (Zarraonaindia et al., 2020). Several members of the *Flavobacteriaceae* family have previously been isolated from soils contaminated with different organic contaminants, such as lindane (Jit et al., 2008), metolachlor (Chen et al., 2018), oil (Chaudhary and Kim, 2017) and hydrocarbons (Szoboszlay et al., 2008). *Flavobacteriaceae* tolerance to lindane could explain its presence in the studied soils. Furthermore, some members of the genus *Flavobacterium* (*Flavobacteriaceae* family) have been related to manure composting processes (Du et al., 2021; Yin et al., 2019; Zhang et al., 2019), as well as leaf and branch compost (Kim et al., 2012), which could explain its significantly increased abundance in composted horse manure-amended soil.

5. Conclusions

Soil microbial parameters were not adversely impacted by nZVI application. The addition of nZVI was the most effective factor regarding the reduction in soil lindane concentrations. The highest reduction in extractable Zn levels was achieved in the simultaneous presence of *B. napus*, nZVI and organic amendments. The combination of the three treatments (rhizoremediation with *B. napus*, nanoremediation with nZVI, biostimulation with organic amendments) led to significantly higher values of soil microbial biomass, activity and biodiversity, thus resulting in an improvement in soil health. We concluded that the combination of remediation approaches can help overcome the limitations of each individual strategy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2022.102621.

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